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Safety and immunogenicity of a recombinant *Plasmodium falciparum* AMA1-DiCo malaria vaccine adjuvanted with GLA-SE or Alhydrogel® in European and African adults: A phase 1a/1b, randomized, double-blind multi-centre trial

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ABSTRACT

Background: *Plasmodium falciparum* Apical Membrane Antigen 1 Diversity Covering (*Pf*AMA1-DiCo) candidate vaccine is a formulation of three recombinant variants of AMA1 designed to provide broader protection against parasites with varying AMA1 sequences.
Methods: In this staggered phase Ia/Ib randomized, double blind trial, healthy French adults received AMA1-DiCo with either Alhydrogel® (n = 15) or GLA-SE (n = 15). Following a safety assessment in French volunteers, GLA-SE was chosen for the phase Ib trial where healthy Burkinabe adults received either AMA1-DiCo/GLA-SE (n = 18) or placebo (n = 18). AMA1-DiCo (50 μg) was administered intramuscularly at baseline, Week 4 and 26.
Results: AMAI-DiCo was safe, well tolerated either with Alhydrogel® or GLA-SE. In European volunteers, the ratios of IgG increase from baseline were about 100 fold in Alhydrogel® group and 200–300 fold in GLA-SE group for the three antigens. In African volunteers, immunization resulted in IgG levels exceeding those observed for the European volunteers with a 4-fold increase. DiCo-specific IgG remained higher 26 weeks after the third immunization than at baseline in both European and African volunteers. Induced antibodies were reactive against whole parasite derived from different strains.
Conclusion: AMA1-DiCo vaccine was safe and immunogenic whatever the adjuvant although GLA-SE appeared more potent than Alhydrogel® at inducing IgG responses.
Clinical Trials Registration. ClinicalTrials.gov NCT02014727; PACTR201402000719423.

1. Introduction

Apical Membrane Antigen 1 (AMA1) is a protein of apicomplexan parasites with an essential role in host cell invasion [1]. The AMA1 ectodomain is an important *Plasmodium falciparum* blood-stage vaccine target and antibodies against the ectodomain have been shown to interfere with AMA1 processing and prevent red cell invasion *in vitro* [2–6]. This effect requires immunization with correctly folded AMA1 [7,8]. The challenge however is the extreme sequence diversity of AMA1. In a single trial site in Mali,

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214 AMA1 variants were identified among 506 subjects [9]. With a database currently at 2372 entries, we have identified 841 unique AMA1 variants and 140 polymorphic amino acid positions in the ectodomain [E. Remarque; unpublished]. The importance of this polymorphism is best illustrated by the outcome of a vaccine trial with a single allele vaccine; overall vaccine efficacy was only 17% whereas efficacy against the homologous (3D7) allele was 64% [10].

The AMA1 Diversity Covering (DiCo) proteins were designed to overcome AMA1 polymorphism. To this end, three artificial protein sequences were constructed incorporating a high degree of variation based on 355 sequences available at the time of design [11]. Immunization with a mixture of three DiCo proteins yielded antibodies capable of inhibiting *in vitro* growth of a panel of AMA1 variants in rabbits and non-human primates [11–13]. Studies in monkeys were done to compare the potency of different adjuvants available at the time (CoVaccine HT). It was subsequently found that continuous access to the patented CoVaccine HT adjuvant was not guaranteed, so we opted for another adjuvant with guaranteed access (GLA-SE). Rabbit studies were therefore performed with GLA-SE and Alhydrogel, but studies were not repeated in monkeys with the adjuvants selected for clinical trials.

For comparative reasons, Alhydrogel® is used as reference adjuvant in European Vaccine Initiative (EVI) malaria phase Ia clinical trials. However, *P. falciparum* AMA1 adjuvanted with Alhydrogel® induced modest (inhibitory) antibody responses in European adults [14–16] and failed to protect Malian children from natural exposure [17]. One possibility was to use the adjuvant AS02 which has an acceptable safety profile and yielded about 4-fold higher antibody levels than Alhydrogel [15]. AS02 contains a squalene emulsion, a TLR4 agonist (MPL), saponin (QS21) and tocopherol. Because AS02 was not available for the AMA1-DiCo trial, it was decided to use another adjuvant GLA-SE which contains a squalene emulsion and a TLR4 agonist (GLA). In addition GLA-SE has a guaranteed continuous access. This proprietary adjuvant (Infectious Disease Research Institute Seattle, IDRI, USA) is a stable oil-in-water emulsion containing glucopyranosyl lipid adjuvant (GLA), a synthetic monophosphorylated lipid, which is a Toll-like receptor 4 agonist.

We conducted a phase Ia trial in French volunteers to evaluate the safety and immunogenicity of three doses of 50 μg AMA1-DiCo formulated with either GLA-SE or Alhydrogel® adjuvant. Following a positive safety assessment of the two first doses in all French volunteers, GLA-SE was chosen for the phase Ib trial. Subsequently we conducted a phase Ib in Burkinabe volunteers to evaluate the safety and immunogenicity of three doses of 50 μg AMA1-DiCo formulated with GLA-SE compared to a saline control. Malaria transmission is seasonal, being low during the dry season (November to May) and high during the rainy season from June to October. During the rainy season, the clinical malaria incidence rate in children 2.4 per child-year at risk in children under five years of age with *P. falciparum* accounting for more than 95% of infections [18]. The main vectors are *An. gambiae* and *An. funestus*. From February to May, the number of bites per person per night (Entomological Inoculation Rate; EIR) due to *An. Gambiae s.l.* was negligible. However, the EIR increased from June to September, decreased from September to November and remained low until the next rainy season. Findings from the same area highlighted that targeting domain I of AMA1 revealed the presence of AMA1 alleles without a statistical overexpression of a particular allele [19].

2. Materials and methods

2.1. Vaccine formulations

The investigational vaccine is AMA1-DiCo formulated with the adjuvant GLA-SE (IDRI, USA) or Alhydrogel® manufactured by Brenntag (Denmark), aseptically diluted at Nova Laboratories Ltd (United Kingdom).

AMA1-DiCo vaccine consists of three highly purified recombinant *P. falciparum* AMA1 Diversity Covering proteins (*Pf*AMA1-DiCo 1, 2 and 3). The sequences of the artificial proteins *Pf*AMA1-DiCo 1–3 are derived from the naturally occurring variants of the AMA1-protein in different parasite strains.

AMA1-DiCo is artificial and the sequences of proteins do not match with any variants from AMA1 in natural parasite population; however, when used as a mixture, the AMA1-DiCo proteins induce antibodies that are broadly reactive with many AMA1 variants [11,20]. Similar observations were made for mixtures of natural alleles [12,21]. We hypothesize that this is due to the fact that strain (DiCo)-specific epitopes are diluted out relative to conserved epitopes [20,21]. No interactions between the three DiCo proteins in rabbit immunogenicity studies other than the relative dilution of strain specific epitopes relative to conserved epitopes have been observed.

Each of the three *Pf*AMA1-DiCo proteins was expressed individually in *Pichia pastoris*, purified, mixed in a 1:1:1 mass ratio and freeze-dried to obtain the AMA1-DiCo lyophilized malaria vaccine [22].

The content of one vial of lyophilized AMA1-DiCo was reconstituted at each trial site pharmacy with 0.3 mL saline for injection. For the Alhydrogel® formulation, 0.3 mL Alhydrogel® was added to one vial of reconstituted AMA1-DiCo. Each 0.5 mL dose contained 50 μg AMA1-DiCo and 0.85 mg aluminum. For the GLA-SE formulation, 0.3 mL of diluted GLA-SE was added to one vial of reconstituted AMA1-DiCo. Each 0.5 mL dose contained 50 μg AMA1-DiCo and 2.5 μg GLA in 2% oil.

2.2. Study design

2.2.1. Fast-track clinical trial strategy

To accelerate early stage vaccine development, EVI has designed a fast-track strategy where the first-in-human evaluation is done through a staggered multicenter phase Ia/Ib clinical trial. This allows proceeding quickly with the immunization of the African volunteers of the phase Ib trial after a review of the safety data by an independent data safety monitoring board of the phase Ia first vaccination dose in European adults. It was planned to test both GLA-SE and Alhydrogel adjuvants in the phase Ia and, in view of the low immunogenicity results observed with AMA1 vaccine adjuvanted with Alhydrogel in previous trials [14–16], to drop the Alhydrogel adjuvant in the phase Ib provided safety of the GLA-SE adjuvant was confirmed in the European volunteers. The control arm of phase Ib has received saline.

This staggered phase Ia/Ib, randomized, double-blind, multicenter trial was designed to assess the safety and immunogenicity of three intramuscular injections of 50 μg AMA1-DiCo administered in the deltoid muscle at Day 0, Week 4 and 26 with either Alhydrogel® or GLA-SE adjuvants. Healthy volunteers were included in the following two cohorts: non-exposed European volunteers in France (Centre Clinique d'Investigation Cochin Pasteur, Hôpital Cochin, Paris, Cohort A, n = 30) and malaria-exposed African volunteers in Burkina Faso (Centre National de Recherche et de Formation sur le Paludisme, CNRFP; Ouagadougou, Cohort B, n = 36).

2.2.2. Participants

Participants were healthy males and non-pregnant females aged 20–45 years. Exclusion criteria for all subjects included: symptoms, physical signs or laboratory values suggestive of systemic disorders; positive HIV, HBV and HCV tests. For European volunteers, additional exclusion criteria were: a history of malaria or travel in malaria endemic areas within the past six months; pos-

itive serology for malaria antigen *Pf*AMA-1; intention to travel to malaria endemic countries during the trial.

Written informed consent was obtained from each volunteer. The protocol was conducted in accordance with the Declaration of Helsinki and International Committee of Harmonization Good Clinical Practice Guidelines and approved by the relevant ethics committees and regulatory authorities of France and Burkina Faso.

2.2.3. Study objectives

The primary objective was to evaluate the safety of AMA1-DiCo vaccine. The secondary objectives were to measure anti-DiCo IgG level responses and IFNγ and IL-5 cytokine production after *in vitro* stimulation with the vaccine antigens. The recognition of the native parasite antigen by the induced antibodies was part of the exploratory objectives.

2.2.4. Study endpoints

The primary endpoint was the assessment of vaccine safety. Volunteers were observed for 60 min after each vaccination. Diary cards were used to record local and systemic reactogenicity occurring during 14 days after vaccination; any unsolicited adverse event between the first vaccination and four weeks after the third vaccination; any serious adverse event occurring from the inclusion throughout the trial. The safety was also assessed during visits one week and four weeks after each vaccination. Volunteers were followed for 26 weeks after the last vaccination.

Safety was also determined by laboratory evaluations (clinical chemistry and hematology) on blood samples collected one and four weeks after each vaccination. The scoring for severity of adverse events is described in Supplementary Table 1.

The secondary endpoint of the study was the assessment of immunogenicity. The immune response was evaluated in a blinded manner at each site using the same procedures in both countries.

2.3. Antibody measurement by ELISA and IFA

Titers of IgG against vaccine antigens (DiCo1, DiCo2 and DiCo3) on samples at Day 0, Week 4, 5, 8, 26, 27, 30 and 52 were measured by ELISA using plates coated with *Pf*AMA1-DiCo1, 2 or 3 antigens as described in Supplementary Elisa test.

All AMA1 variants are serologically cross reactive; about 50% of antibodies induced by monovalent immunisation cross react with other variants [11,23]. Both natural AMA1 variants and DiCo's yield similar IgG titres with endemic sera. The pool serum used for the ELISA had IgG titres ranging from 120 to 160 μg/mL to seven AMA1 variants tested (DiCo's, FVO, CAMP, HB3 and 3D7). As the DiCo proteins are the immunogens, the immune responses were separately assessed for each antigen rather than for the three antigens simultaneously.

As exploratory endpoint, IFA was performed on cultured NF54 or FCR3 *P. falciparum* parasitized red blood cells, basically as described before [17], using dilutions 1:25, 1:50, 1:100 for NF54 and up to 1:200 for FCR3.

2.4. IFNγ and IL-5 production by ELISPOT

The cellular immune response was assessed *in vitro* by measuring production of the T-cell IL-5 and IFNγ cytokines by ELISPOT after stimulation with the vaccine antigens on samples obtained at Day 0, Week 26, 30 and 52. As previously detailed [24], a negative (unstimulated cells) and a positive control (cells stimulated with PMA-Ionomycin) were included for each subject. A response was considered positive if the number of spots in the wells stimulated with the vaccine antigen was twofold higher than the number of spots in the negative control using a cutoff of 10 SFC/10^5 cells after background subtraction.

2.5. Statistical methods

It was estimated that a group size of 15 subjects would give a minimum power of 80% for detecting one or more serious adverse events that occurred with a frequency of at least 10%.

Randomization lists were generated with a 1:1 ratio using random block sizes of 2 and 4, independently by the trial statistician. A treatment number linked to the vaccine was provided on-line. The pharmacists prepared the vaccine doses. Volunteers, investigators and laboratory personnel were blinded to vaccine assignment.

The safety population included all volunteers who received at least one injection (Intent-to-treat population, ITT). The immunogenicity analysis included volunteers who received the three doses of the allocated product according to protocol (Per-protocol population, PP). The statistical analyses were merely exploratory since the study was not designed to demonstrate statistically significant differences.

3. Results

3.1. Study population

The first immunization was in January 2014 in France and in July 2014 in Burkina Faso. Last visits occurred in March and July 2015 in France and Burkina Faso, respectively.

Among 41 screened European volunteers (cohort A), 30 fulfilled the inclusion criteria. The most common reasons for exclusion were positive serology for malaria antigens (n = 4), significant laboratory abnormalities (n = 2) (Fig. 1). Volunteers were randomized either to the AMA1-DiCo plus Alhydrogel® group (n = 15) or the AMA1-DiCo plus GLA-SE group (n = 15).

The ITT population includes 30 volunteers analyzed for safety while the PP population includes 24 volunteers analysed for immunogenicity. The total number of immunizations in cohort A was 44 in the Alhydrogel® group (15, 15 and 14 for the first, second and third vaccination, respectively) and 37 in the GLA-SE group (15, 12 and 10 for the first, second and third vaccination, respectively).

Among 78 screened African volunteers (cohort B), 36 fulfilled the inclusion criteria. The most common reason for exclusion was significant laboratory abnormalities (n = 21). Four subjects were not included as the target sample size was reached. Volunteers were randomized either to AMA1-DiCo plus GLA-SE (n = 18) or placebo (n = 18).

The ITT population includes 36 volunteers analyzed for safety while the PP population includes 33 volunteers analyzed for immunogenicity. The total number of immunizations in the cohort B was 51 in the GLA-SE group (18, 17 and 16 for the first, second and third vaccination, respectively) and 53 in the placebo group (18, 18 and 17 for the first, second and third vaccination, respectively).

The baseline demographic characteristics of the four groups were comparable (Table 1). There was 60% of women in cohort A and 73% in cohort B.

3.2. Safety

There was no vaccine-related serious adverse event during the trial.

3.2.1. Safety results for cohort A (France)

Unrelated adverse events until one month after the last immunization included one serious adverse event after the first immunization (epilepsy; the volunteer did not receive further

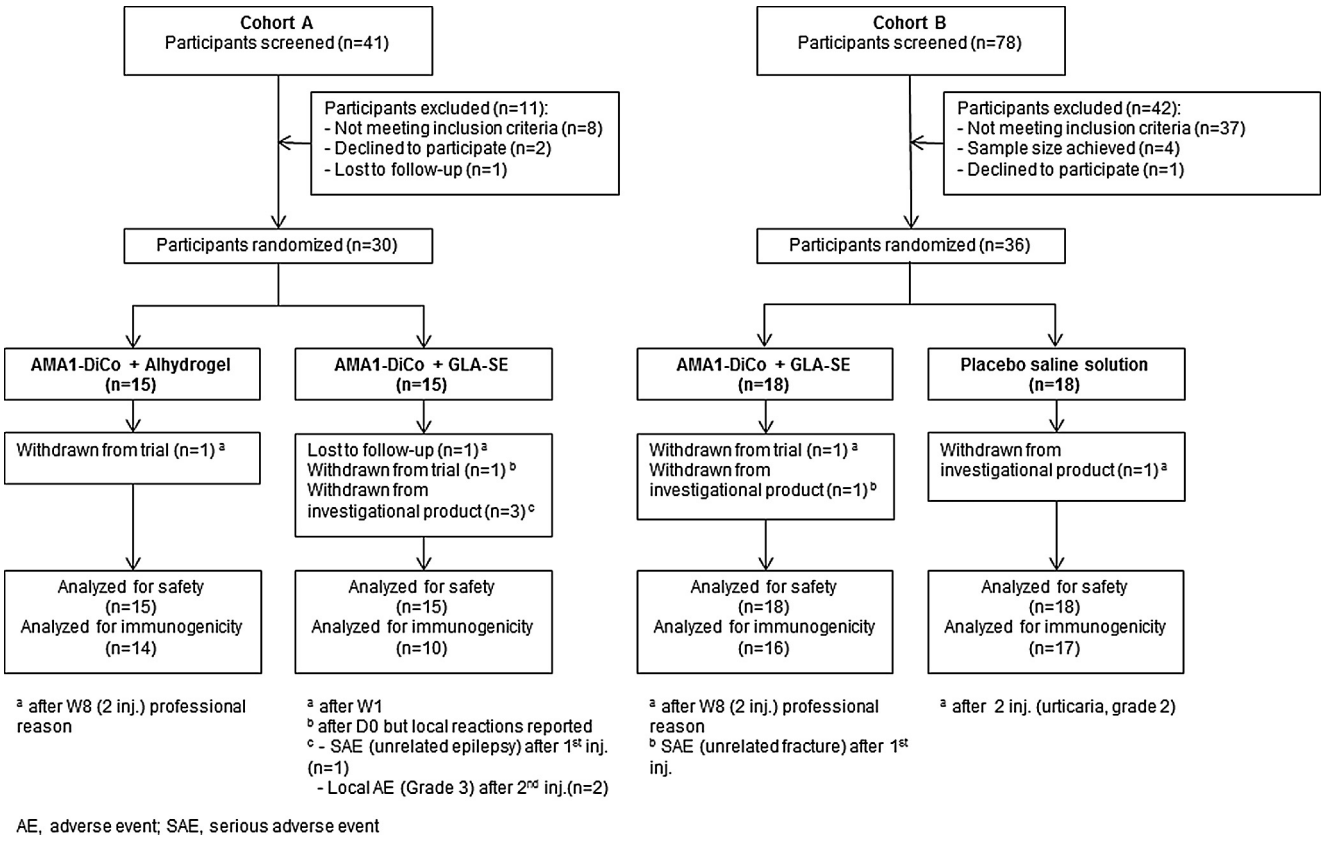

Fig. 1. Study flow chart. Cohort B (Burkina Faso) enrolment was initiated following safety assessment of the first vaccine dose (confirmed by the second dose) in all participants in cohort A (France).

Table 1
Baseline demographics.

	European cohort		African cohort	
	AMA1-DiCo + Alhydrogel® n = 15	AMA1-DiCo + GLA-SE n = 15	AMA1-DiCo + GLA-SE n = 18	Placebo n = 18
Number of Females	10	8	12	14
Age, years				
Mean	29.9	29.7	30.8	36.0
Range	21.5–44.4	21.6–43.8	21.1–44.1	24.5–44.6
Weight, kg				
Mean	68.0	72.0	60.5	60.8
Range	52–89	53–85	43.7–67.5	50.0–69.8
Body mass index, kg/m²				
Mean	22.6	24.1	22.1	21.6
Range	18–28.4	19.1–29.4	15.7–24.5	19.8–28.4

immunizations). Subjects with adverse reactions from day 0 through day 14 after immunization were 14 (93%) in the Alhydrogel® group and 15 (100%) in the GLA-SE group respectively: 14 (93%) and 15 (100%) reported local reactions and 8 (53%) and none reported systemic reactions, respectively.

Two volunteers in the GLA-SE groups experienced local reactions after the second immunization leading to withdrawal for Grade 3 induration (60 mm) and swelling (60 mm) for one volunteer and Grade 3 redness (55 mm), induration (55 mm) and local warmth for the other.

Local event were reported in the Alhydrogel® and GLA-SE groups by 87% (13/15) and 100% (15/15) of volunteers after the first injection, 80% (12/15) and 92% (11/12) after the second injection, 57% (8/14) and 80% (8/10) after the third injection, respectively (Fig. 2). The most frequent local reactions were pain at injection site and limitation of arm motion abduction at shoulder. All events were either Grade 1 or 2 and resolved without any sequela.

Systemic events, were reported in the Alhydrogel® and GLA-SE groups by 47% (7/15) and 0% (0/15) of the volunteers after the first injection, 20% (3/15) and 0% (0/12) after the second injection, 14% (2/14) and 0% (0/10) after the third injection, respectively. Only four Grade 2 events (headaches) were reported, all in the Alhydrogel® group.

3.2.2. Safety results for cohort B (Burkina Faso)

Adverse reactions from day 0 through day 14 after immunization were reported in 13 (72%) in the GLA-SE group and 12 (67%) in the placebo group: 11 (61%) and 8 (44%) reported local reactions and 7 (39%) volunteers in both groups reported systemic reactions.

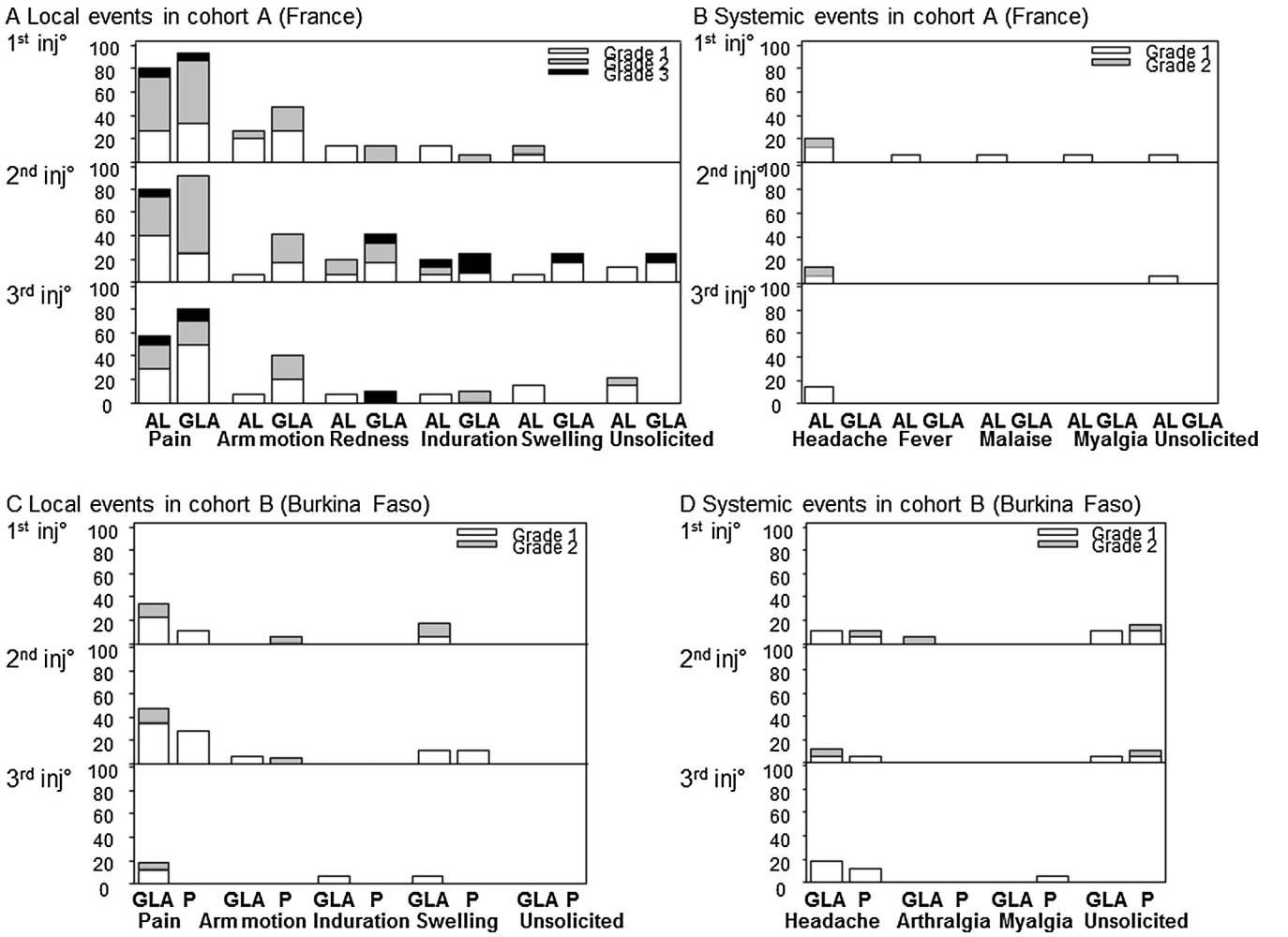

Fig. 2. Solicited and unsolicited reports of adverse events 14 days after the first, second and third injection of the investigational products (AL: AMA1-DiCo/Alhydrogel®, GLA: AMA1-DiCo/GLA-SE; P: placebo). In cohort A (A and B), unsolicited local reactions in the vaccine group AMA1-DiCo/Alhydrogel® after the second injection: ecchymosis, hematoma and after the third injection: pruritus, pain; unsolicited local reactions in the vaccine group AMA1-DiCo/GLA-SE after the second injection: hemorrhage, pruritus, injection site warmth (Grade 3). Unsolicited systemic reactions in the vaccine group AMA1-DiCo/Alhydrogel® after the first injection: hot flush and after the second injection: feeling drunk. In cohort B (C and D) unsolicited systemic reactions in the vaccine group AMA1-DiCo/GLA-SE after the first injection: fall, fever, vertigo and after the second injection: ear pain; unsolicited systemic reactions in the placebo group after the first injection: torticollis, urticaria (Grade 2) and after the second injection: torticollis (Grade 2) and urticaria. Percentage of volunteers with vaccine-related adverse events are reported with severity grading.

Urticaria was reported in the placebo group after the first and second immunization and led to withdrawal. Unrelated adverse events one month after the last immunization included one serious adverse event (radius fracture).

Overall, in the GLA-SE and placebo groups, local events were reported by 33% (6/18) and 17% (3/18) after the first injection, 53% (9/17) and 44% (8/18) after the second injection, 19% (3/16) and 0% (0/17) after the third injection. The most frequent reactions were pain at injection site and swelling (Grade 1 or 2) (Fig. 2).

Systemic events were reported in the GLA-SE and placebo groups by 22% (4/18) and 22% (4/18) after the first injection, 18% (3/17) and 17% (3/18) after the second injection, 19% (3/16) and 12% (2/17) after the third injection, respectively. The most frequent systemic events were headaches with only one Grade 2 headache in the GLA-SE group.

3.3. Humoral immune response

3.3.1. Cohort A IgG titers

The European volunteers showed comparable geometric means of baseline IgG titers in the two adjuvant groups (0.2 μg/mL) for the three antigens (Fig. 3). Peak responses in the Alhydrogel® group were obtained at Week 30, four weeks after the third injection, whereas peak responses in the GLA-SE group were obtained at Week 27, one week after the third vaccination. The responses at Week 30 for the three antigens were 2-fold higher in the GLA-SE group as compared with the Alhydrogel® group with geometric mean IgG concentrations ranging from 37.7 to 60.2 μg/mL and from 19 to 22.9 μg/mL, respectively (Supplementary Table 2). The changes in IgG concentrations over time relative to the baseline value are reported as fold increases in Fig. 4. Four weeks after the third injection, ratios were about 100 fold in the Alhydrogel® group, and 200–300 fold in the GLA-SE group. At Week 52, antibody levels to all DiCo antigens remained elevated over baseline, as shown by geometric means and 95% confidence intervals; 27.7 to 32-fold for the Alhydrogel® group and 51.4 to 70.1 for the GLA-SE group (Fig. 4). Both formulations of vaccine were immunogenic although GLA-SE appeared more potent than Alhydrogel® at inducing IgG responses to the three DiCo antigens.

3.3.2. Cohort B IgG titers

At baseline, both groups of African volunteers showed geometric mean IgG concentrations of 20–30 μg/mL for the three DiCo antigens (Fig. 3). In the GLA-SE group the geometric mean IgG con-

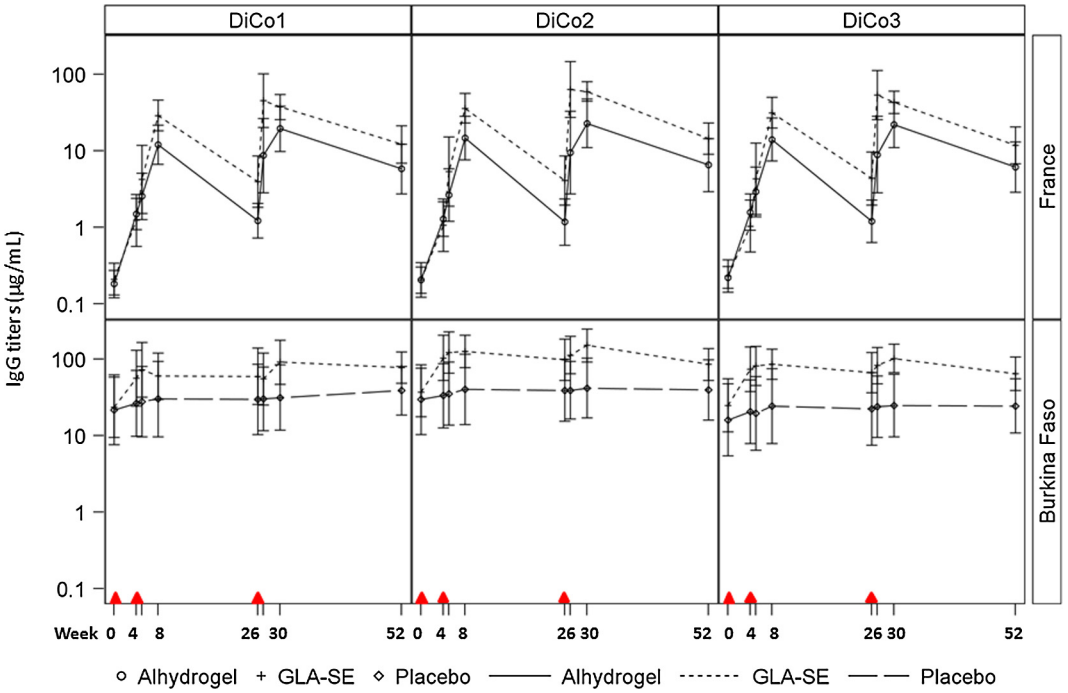

Fig. 3. Humoral IgG response to the AMA1-DiCo antigens (DiCo1, DiCo2, DiCo3) per group at Weeks 0, 4, 5, 8, 26, 27, 30, 52 in cohort A (France; AMA1-DiCo/Alhydrogel® and AMA1-DiCo/GLA-SE groups; top row) and in cohort B (Burkina Faso; AMA1-DiCo/GLA-SE and placebo groups; bottom row) in per-protocol populations. Arrows indicate immunization time points (Week 0, Week 4, and Week 26). IgG titers geometric means are given with 95% confidence intervals represented by the range bars.

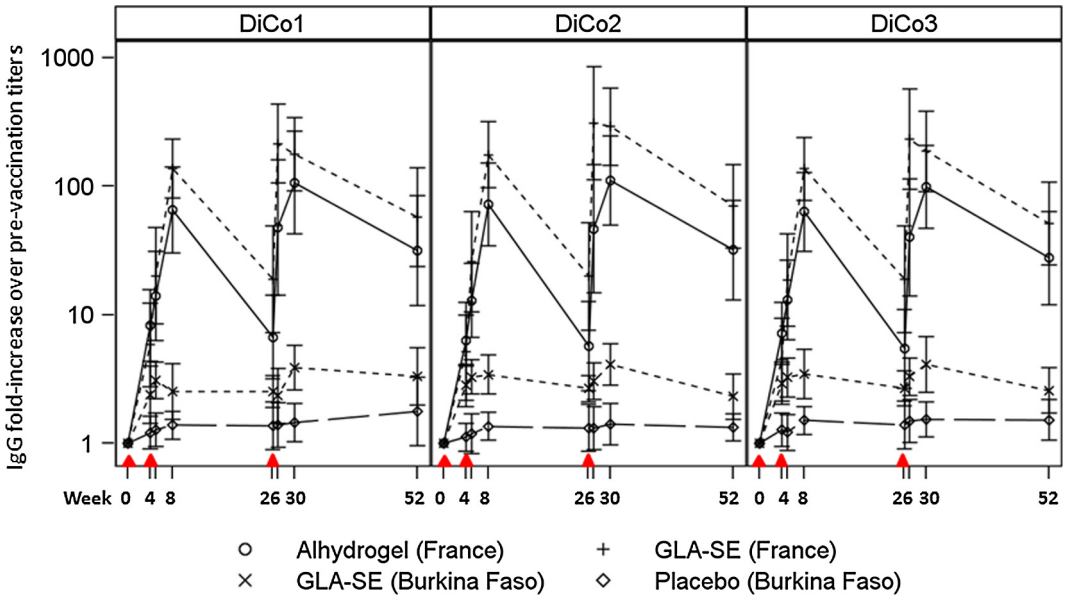

Fig. 4. IgG fold-increase over pre-vaccination titers to the AMA1-DiCo antigens (DiCo1, DiCo2, DiCo3) per group at Weeks 0, 4, 5, 8, 26, 27, 30, 52 in cohort A (France; AMA1-DiCo/Alhydrogel® and AMA1-DiCo/GLA-SE groups) and in cohort B (Burkina Faso; AMA1-DiCo/GLA-SE and placebo groups) in per-protocol populations. Arrows indicate immunization time points (Week 0, Week 4, and Week 26). Continuous lines represent the AMA1-DiCo/ Alhydrogel® group, dashed lines represent AMA1-DiCo/GLA-SE groups, and wide dashed lines represent the placebo group. Fold-increases over the Day 0 geometric mean are given with 95% confidence intervals represented by the range bars. Pre-vaccination (Day 0) IgG concentrations for the four study groups are given in Table 2 (supplementary material).

centrations increased to 90–150 µg/mL at Week 30 with wide confidence intervals showing the spread of those results.

For the GLA-SE group increases in IgG concentration over baseline were already evident by Week 4 just before the second immunization and, by Week 30, four weeks after the third injection, the fold IgG increase ranged from 3.9-4.1 indicating that the three AMA1-DiCo antigens, when administered with GLA-SE, induced an immune response in malaria-exposed volunteers (Fig. 4).

3.3.3. IFA results

All the 24 French volunteers were positive in IFA for either FCR3 or NF54, 22 were positive in IFA on the FCR3 strain parasites, while 20 out of 24 were positive on NF54 (3D7). Most volunteers were positive at the 1:200 dilution for FCR3, 8/14 and 9/10 in Alhydrogel® and GLA-SE groups respectively and the 1:100 dilution for NF54 showed highest positivity frequencies, 5/14 and 3/10 in Alhydrogel® and GLA-SE groups respectively.

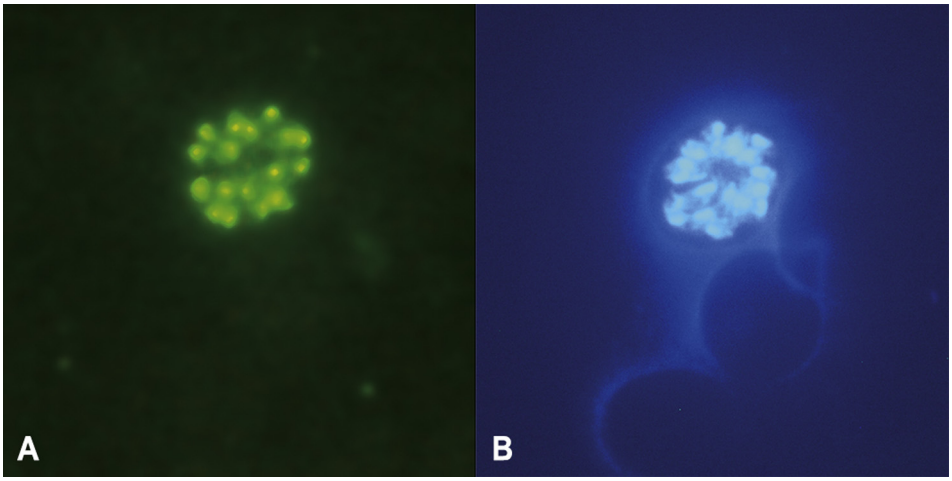

Fig. 5. Representative immunofluorescent microscopy picture, showing recognition of native PfAMA1 antigen (FCR3-strain) on merozoites by induced anti-AMA1 antibodies (A). DAPI staining in (B). Merozoites were incubated with 100× diluted serum from an immunized French volunteer, four weeks after the third and final immunisation. Secondary staining was with Rabbit anti-human immunoglobulin conjugated to FITC. Photo was taken at magnification of 1000×.

On average the highest dilution for which the volunteers were positive was lower for NF54 compared to FCR3. Sera from the day 1 visit of the French volunteers were all negative in IFA, while four sera from Burkinabe volunteers that were tested stained strongly positive in IFA (FCR3) in a 1:400 dilution, both at Day 1 and Week 30, 4 weeks after the last immunization. Three of the four African sera showed some slight apical staining at Week 30, albeit not strong (data not shown). Fig. 5 shows the typical apical staining, as described for PfAMA1 in a French volunteer.

3.4. Cellular immune response

Volunteers immunized with the vaccine antigen adjuvanted with Alhydrogel® displayed a Th2-type T-cell response as indicated by an elevated spot forming cells detected by an IL-5 ELISPOT assay when PBMC were stimulated with DiCo1, DiCo2, and DiCo3 antigens. This response peaked at Week 30 and was observed in more than 50% of volunteers. No IFN-γ T-cell response was observed in the same group of volunteers. Surprisingly, neither cohort A nor B volunteers immunized with vaccine adjuvanted with GLA-SE had detectable Th1- or Th2-biased T-cell responses, as measured by either the IFN-γ or the IL-5 ELISPOT assay (Fig. 6).

4. Discussion

In this study, AMA1-DiCo vaccine, adjuvanted with Alhydrogel® or GLA-SE, was well tolerated both in malaria-naïve Europeans and in African volunteers previously naturally exposed to malaria infections. The fact that grade 3 adverse reactions occurred in malaria-naïve population, but not in endemic population remains unclear. However, due to the small sample size of each cohort, extrapolation to larger trials should be carried out with caution.

In European volunteers not previously exposed to malaria, GLA-SE appeared to be a more potent adjuvant than Alhydrogel® at inducing an IgG response to the three DiCo antigens, and the decision to proceed in Burkina Faso with only GLA-SE was well anticipated. In African volunteers, previous exposure to malaria was evidenced by high baseline antibody levels to the three antigens. The baseline IgG titers were frequently in the same range as those achieved in the European cohort following vaccination. Nevertheless, IgG levels of the African GLA-SE group further increased after vaccination. Thus, one month after the third injection, mean IgG levels as high as 92.2–152.2 μg/mL were reported in the GLA-SE group as compared to 24.9–42.3 μg/mL in the placebo group or to 37.7–60.2 μg/mL in the GLA-SE group of the European cohort. The AMA1-DiCo-specific IgG level increase from baseline in the vaccinated African cohort was 3.9–4.1-fold after the third injection and increased IgG levels persisted at least six months after the third injection. There was a trend towards a slightly higher response to DiCo-2 than DiCo-1 and DiCo-3 in the GLA-SE groups, but not in the French Alhydrogel® group. This difference may be due to intrinsic protein properties and was already observed in rabbit studies [11] or may be an interaction between adjuvant and antigen.

The three DiCo protein sequences differ from naturally occurring variants, but are serologically about 50% cross reactive [11,12] Similarly natural variants show 50% serological cross reactivity [20,23] When used as a mixture DiCo proteins induce an increased fraction of cross reactive antibodies thus increasing breadth [12].

In preclinical studies, AMA1-DiCo induced high levels of AMA1 antibodies capable of recognizing both natural and DiCo variants. The antibodies induced were also functional, i.e. had GIA activity [13]. Of note potent adjuvants (Montanide ISA and Covaccine HT) with an important impact on vaccine immunogenicity were used in the non-human primate studies. In contrast, in the current trial, it seems that GLA-SE was not as potent as expected. This difference between adjuvants of *Pf*AMA1-DiCo is supported when comparing its immunogenicity with Alhydrogel versus AS02 or GLA-SE in healthy volunteers [15]. Moreover, IgG levels to AMA1-DiCo were about half those observed in the trial of Thera et al. [10]. This may be due to different vaccine regimens, adjuvants or populations.

Vaccination with AMA1, either purified from merozoites or produced as recombinant protein, has protected primates from malaria [6,13,25,26]. Passive transfer of antibodies against AMA1 has protected mice against malaria [1]. In addition, AMA1 appears to be immunogenic in populations naturally exposed to malaria infection. Since the protective effect of the vaccine is expected to be mediated by antibodies, high levels of IgG elicited in the European and also in the malaria-exposed African subjects are promising results for the AMA1-DiCo vaccine. Additionally, IFA positive signals for both the NF54 and FCR3 parasites strains add to these encouraging results.

In previous clinical trials of subunit vaccines adjuvanted with GLA-SE [27,28], an IFNγ T-cell response detected by ELISPOT was reported, which contrasts with our results. However, the vaccine's protein dosage [27] and the ELISPOT positivity criteria used [28]

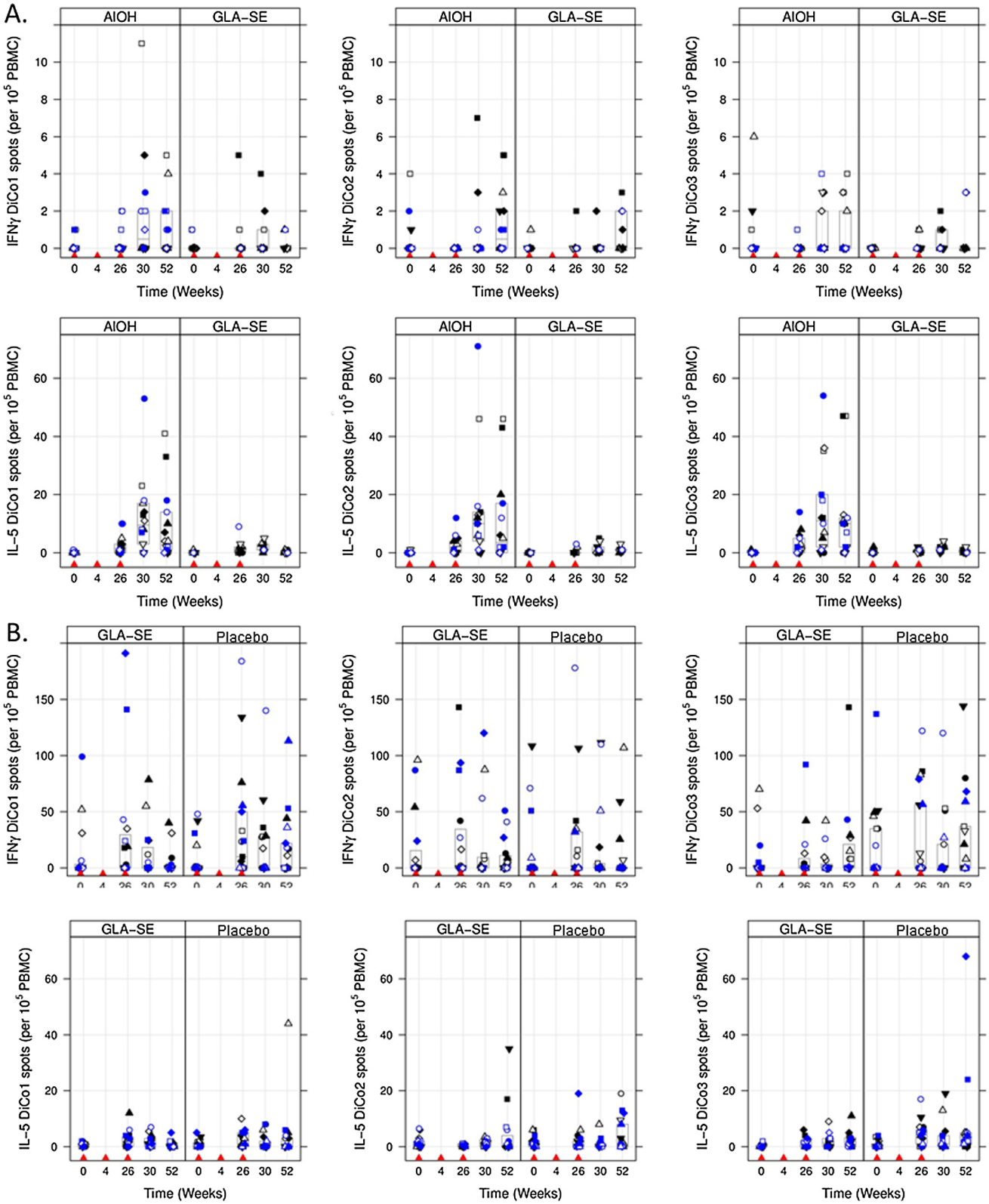

Fig. 6. ELISPOT assay in in cohort A (France; AMA1-DiCo/Alhydrogel® and AMA1-DiCo/GLA-SE groups) and in cohort B (Burkina Faso; AMA1-DiCo/GLA-SE and placebo groups) in per-protocol populations for IFN γ and IL-5 after stimulation with AMA1-DiCo antigens. Production of IFN γ and IL-5 was measured by counting spots in ELISPOT plates per 10^5 cells. Values for each subject within a vaccine group are indicated by a specific symbol (same symbol within each group is same subject throughout graphs), boxes indicate median and quartile ranges. Vaccine groups are indicated by Adjuvant name (AlOH = Alhydrogel, GLA-SE) or Placebo.

differed among the various trials. Recent studies in mice showed that IFN-γ-producing CD4+ T cells were not required for the efficacy of vaccine against tuberculosis [29].

Correlation analyses on ELISpot data and IgG titres observed at week 30 in the French subjects did not show any significant correlation between IgG titre and ELISpot counts. It can be argued, how-

ever, that T cell help has been provided as exemplified by the presence of IgG titres to the vaccine antigens. Moreover, the Burkinabe subjects (both vaccinated and placebo groups) do show significant IFN-g responses following stimulation with the vaccine antigens, suggesting that the assay was technically sound.

The IgG levels observed for the individual DiCo antigens thus can be considered indicative for vaccine take.

A previous dose-escalating phase Ia trial evaluated the safety and immunogenicity of recombinant *Pf*AMA1 produced in *Pichia pastoris* with different adjuvants in European volunteers not previously exposed to *P. falciparum* [15]. Volunteers who received three injections of 50 μg of *Pf*AMA1 in combination with Alhydrogel® achieved lower IgG titers than observed in the present study with AMA1-DiCo vaccine. The possibility that the use of the three proteins *Pf*AMA1-DiCo 1, 2 and 3 could offer better protection compared with a single unique protein remains to be established in further studies.

In conclusion, the AMA1-DiCo vaccine antigen was safe and immunogenic with both adjuvants and GLA-SE appeared to be more potent than Alhydrogel® in inducing a large and long-lasting IgG response. AMA1-DiCo/GLA-SE induced an immune response to the three related apical membrane antigens in malaria-exposed volunteers. In the event AMA1 DiCo vaccine is capable of inducing broadly reactive antibodies, this would support future development. Moreover the data suggest that more potent adjuvants should be used.

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Conflict of interest

Three of the authors (BF, CK and ER) are on a patent (Patent 8741305 (Jun 3, 2014)), "Protein Composition for Inducing an Immune Response in a Vertebrate Comprising a Plurality of Protein Variants Covering the Heterogeneity of a Single Antigen, AMA1" regarding the design of the proteins comprised in the product under investigation. SH reports grants from Dutch Ministry of Foreign Affairs, Directorate-General for International Cooperation, grants from Irish Aid during the conduct of the study.

All authors have submitted the ICMJE Form for disclosure of Potential Conflits of Interest.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.09.027.

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